Healing kinetics of oral soft tissue wounds treated with recombinant epidermal growth factor: Translation from a canine model

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Abstract: OBJECTIVE To test whether or not topically administered recombinant human epidermal growth factor (rhEGF) accelerates the early healing phase of oral soft tissue wounds. METHODS One day following the creation of palatal defects (n = 6/animal), 14 dogs were allocated to one of the following five groups: spontaneous healing (SH), vehicle ointment (V), vehicle ointment + rhEGF at concentrations of 1 g/g (EGF1), 10 g/g (EGF10) or 50 g/g (EGF50). Topical administration of ointments was repeated twice per day until sacrifice at days 8 and 16. Wound area was clinically monitored. Keratinocytes proliferation (Ki67-immunolabelling), inflammatory response (IR) and areas of collagen (C) and granulation tissue (GT) were histologically measured. Kruskal-Wallis test with Dunnett correction was used for multiple group statistical comparisons. RESULTS Clinically, in comparison with SH, a significantly smaller wound area was observed in groups EGF1 and EGF10 at day 8 ($p < 0.05$). At day 16, wound closure reached 97.8% in group EGF1 compared to 83.2% in group SH, albeit no statistically different. Histologically, at day 8, significantly more GT was observed in group EGF10 compared to all other groups ($p < 0.05$). At day 16, in addition to a higher Ki67-immunolabelling, groups EGF1 and EGF10 demonstrated a significant decrease in GT and IR with more deposition of C compared to the other groups ($p < 0.05$). CONCLUSION Application of rhEGF enhanced the early healing of acute oral soft tissue wounds compared to SH, predominantly at concentrations of 1 and 10 g/g.

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TITLE PAGE

Healing kinetics of oral soft tissue wounds treated with recombinant epidermal growth factor: translation from a canine model

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ABSTRACT

Objective: To test whether or not topically administered recombinant human epidermal growth factor (rhEGF) accelerates the early healing phase of oral soft tissue wounds.

Methods: One day following the creation of palatal defects (n=6/animal), 14 dogs were allocated to one of the following five groups: spontaneous healing (SH), vehicle ointment (V), vehicle ointment + rhEGF at concentrations of 1μg/g (EGF1), 10μg/g (EGF10) or 50μg/g (EGF50). Topical administration of ointments was repeated twice per day until sacrifice at days 8 and 16. Wound area was clinically monitored. Keratinocytes proliferation (Ki67-immunolabelling), inflammatory response (IR) and areas of collagen (C) and granulation tissue (GT) were histologically measured. Kruskal-Wallis test with Dunnett correction was used for multiple group statistical comparisons.

Results: Clinically, in comparison with SH, a significantly smaller wound area was observed in groups EGF1 and EGF10 at day 8 (p<0.05). At day 16, wound closure reached 97.8% in group EGF1 compared to 83.2% in group SH, albeit no statistically different. Histologically, at day 8,

significantly more GT was observed in group EGF10 compared to all other groups (p<0.05). At day 16, in addition to a higher Ki67-immunolabelling, groups EGF1 and EGF10 demonstrated a significant decrease in GT and IR with more deposition of C compared to the other groups (p<0.05).

Conclusion: Application of rhEGF enhanced the early healing of acute oral soft tissue wounds compared to SH, predominantly at concentrations of 1μg/g and 10μg/g.

CLINICAL RELEVANCE

Scientific rationale for study: Epidermal growth factor demonstrated to facilitate primary and secondary wound healing of the skin. This study aimed to clinically and histologically verify the effect of topical administration of recombinant human epidermal growth factor (rhEGF) on the early healing of open oral soft tissue wounds.

Principal findings: In comparison to spontaneously healing wounds, rhEGF not only increased the wound closure rate during the first week, but also significantly improved the quality of the wound healing at 16 days.

Practical implications: RhEGF accelerated the healing of oral soft tissue wounds.

1- INTRODUCTION

Oral soft tissue repair is characterized by either a primary or a secondary intention healing. Primary intention healing results when the edges of the wound are closely approximated while in secondary intention healing, the wound edges remain distant(Polimeni *et al.,*2006). Oftentimes, excising diseased gingival tissue, curetting root surfaces, avulsing hopeless teeth, or apically positioning the flapped tissues are prevalent clinical scenarios where wound repair exclusively rely upon secondary intention healing(Harrison *et al.,*1991). Open wounds heal *de facto* with the same basic process of inflammation, proliferation and remodelling as do closed wounds. However, each sequence of the secondary healing wounds takes longer to be

completed. A factor of healing delay is the considerable amount of granulation tissue necessary to bridge the distant wound edges in the setting of open injuries(Clark *et al.,*1996).

To accelerate the repair of soft tissue wounds, regenerative medicine approaches have been applied including growth factors used alone or in combination(Kaigler *et al.,*2006). Preclinical and clinical studies resonate that growth factor-driven treatments are helpful boosting the innate healing potential(Schliephake *et al.,*2002). Prominent among these is the strategy whereby epidermal growth factor(EGF) is engaged in productive skin repair(Brown *et al.*,1989; Bodnar *et al.,*2013). As the first growth factor to be isolated, and one of the first to be synthesised using recombinant DNA technology, EGF has been extensively investigated in dermal wound healing(Brake *et al.,*1984;Hong *et al.,*2006;Tanaka *et al.,*2005). According to recent systematic reviews, topical interventions using recombinant human EGF (rhEGF) in clinical settings are robust protocols to promote healing of cutaneous aberrations such as diabetic ulcers or burn injuries(Martí-Carvajal *et al.,*2010;Zhang *et al.,*2014).

In a similar fashion, this potential of EGF has also captured scientific interest with the endeavour to achieve periodontal regeneration. The available data suggest the promise of EGF purposed at improving oral wound healing. To recapitulate, stimulation of proliferation and migration of oral keratinocytes, stimulation of gingival fibroblasts mitogenesis and increase of collagen synthesis(Kim *et al.,*2011;Pansani *et al.,*2017;Ramineni *et al.,*2015) are among the cellular events through which exogenous EGF is harnessed to facilitate the wound repair in vitro. Nevertheless, whether EGF-based treatment is able to facilitate the repair of acute injuries healing by secondary intention remains unknown. In vivo data are missing in terms of (i) the early phases of open oral soft tissue wounds treated with rhEGF and, (ii) possible effects of dosage differential on wound repair.

The aim of this study was to test, in an animal model of acute open oral mucosa wound, whether or not topical application of rhEGF can improve early wound healing compared to spontaneous healing based on clinical and histological analyses.

2- MATERIALS AND METHODS

2.1- Animals

Fourteen 1-year old, 10kg-weighing beagle dogs were used for the purpose of this experimental study, conducted following approval by the Institutional Animal Care and Use Committee, Cronex, Hwasung, Korea(CRONEX-IACUC 201701001).

All surgical and follow-up interventions were performed under general anaesthesia in an operating room and under sterile conditions. General anaesthesia was induced by the injection of tiletamine/zolazepam(0.1mg/kg)(Zoletil, France), xylazine(2.3mg/kg)(Rompun, Korea), and atropine sulfate(0.05mg/kg)(Jeil, Korea). Further analgesia was given for 3 days following surgical procedures(Zipan, Korea). Throughout the experiment period, all animals were housed individually and had ad libitum access to water and laboratory soft dog-food.

2.3- Experimental procedures

2.3.1- Agents

Semi-solid preparations were used for the application on the oral mucosa of rhEGF(Daewoong, Korea) adjusted to concentrations of 1μg/g, 10μg/g and 50μg/g. Gelatin, pectin and sodium carboxymethylcellulose plasticized in hydrocarbon gel(Plastibase; Contract Pharmaceuticlas, Canada) was used as a carrier of rhEGF.

2.3.2- Surgical procedures

A total of 84 soft tissue wounds were experimentally created in 14 animals(n=6/animal; Supplement Table.1) by adapting a previously reported defect model of the masticatory mucosa of the palate(Thoma *et al.*,2012,2016; Ayvazyan *et al.,*2011)(Fig.1a,b).

10mm–diameter cylindrical wounds were obtained by harvesting oral soft tissue using a biopsy punch(Acuderm, USA) inserted at a predetermined depth of 3mm from the surface of the palatal

ridges, leaving the periosteum and part of the submucosa exposed. In order to standardize surgical procedures, individual stents with six 10mm-diamter circular openings served as a guide to symmetrically allocate three equidistant wounds(4mm) to each hemi-palate(Fig.1a).

2.3.3- Treatment of oral mucosa defects

At day1-post-surgery, the following treatment modalities were randomly applied to the dogs following an incomplete block design, where animals were considered as experimental units and defects(six per dog) as subunits(Supplement Table.1):

-*Spontaneous healing:* No ointment administered(negative control group; ***SH***; N=3 dogs, n=18 defects).

-*Topical application of vehicle*(control group control group; ***V***; N=2 dogs, n=12 defects).

-*Topical application of rhEGF:* three concentrations were tested(1µg/g(***EGF1***), 10µg/g(***EGF10***), 50µg/g(***EGF50***); N=3 dogs, n=18 defects for each concentration)

In order to facilitate topical interventions, additional individual stents were prepared with moulding sheets(Durasoft, Germany) deprived from any medicinal properties(Fig.1c). For each defect, 240 mm^3 of the ointment was transferred to the stent, which was then applied on the upper jaw. After 10 minutes, the stent was removed, and the ointment was wiped off from the palatal mucosa.

Administration was repeated twice per day with a 9h-interval(9am, 6pm) in each dog by laboratory workers blinded to the treatment agents from day 1 post-surgery until animals were euthanized. In the group SH, the same procedure was performed while no ointment was applied on the stent

2.3.4- Follow-up

Before surgery, immediately after surgery and at post-surgery day(PSD) 4, 8, 12, and 16, impressions and clinical photographs were taken. Polyether impressions(Impregum F, Germany) of the upper jaws were taken using prefabricated individualized trays(Schmitt *et al.*,2016).

Standardized clinical photographs were taken at an angle of 90◦ to the upper jaw and a distance of 80cm using identical resolution, exposure and enlargement(Rennert *et al*.,2009)

2.3.5- Biopsies and histological processing

At PSD8 and 16, animals were sacrificed by an intravenous injection of potassium chloride (Jeil, Korea) under general anaesthesia. Full thickness mucosa was gently separated from the palatal bone using a periosteal elevator and tissue specimens were separated. Each biopsy was fixed in 10% neutral-buffered formalin solution and embedded in paraffin. The tissue blocks were sectioned in a frontal plane at the most central aspect of the wounds and 5µm-thickness serial slices were cut using a microtome. Three consecutive slides from the center of the wounds were selected and prepared for staining(Ayvazyan *et al.,*2011). The slides were scanned on a high throughput Panoramic Scan(3DHistech, Hungary) and each section image was visualized using Case viewer(3DHistech, Hungary).

2.4- Analyses

2.4.1- Clinical photograph analysis

Photographical recordings of the wounds(before and immediately after surgery, PSD4, 8, 12 and 16) were imported into a software program(ImageJ, NIH, USA) to calculate the surface area covered by unkeratinized tissue(*Wound Area*) expressed in mm^2(Rennert *et al.*,2009)(Fig.1e).

2.4.2- Three-dimensional analysis of the wounds

For volumetric evaluation of tissue repair, impressions obtained before surgery, directly after surgery, and at PSD4, 8, 12, and 16 were poured out(Dentona, Germany). Retrieved casts were optically scanned using an industrial scanner(ATOS II SO4, Germany) and a software(ATOS, Germany) that allowed superimposing the three-dimensional data of the virtual dental models(Schmitt *et al.*,2016)(Fig.1f).

Regions of interest(ROI_V) were individually set by precisely selecting the wound margins in each defect from dental models obtained at the different intervals form baseline surgery for comparative measurements. The variations of tissue volume(Integrated distances in mm^3, Schmitt *et al.*,2016) was thereafter calculated.

2.4.3- Histological analyses

Following staining with H&E and Masson's Trichrome, wound remodelling, inflammation and angiogenesis were histologically assessed by quantitative image analysis of the following parameters(Fig.1g):

-***New Epithelium and Wound Gap***: The length of the epithelium covering the wound's connective tissue(*New epithelium*; µm) and the length of line segment between defect's margins (*Wound gap*; µm)were calculated(Lemo *et al.*,2010).

-***Granulation Tissue and Collagen Areas***: A rectangular region of interest(ROI_1) encompassing the central superficial area of the wound was defined to semi-automatically quantify granulation tissue and collagen formation using a digital image software program(Photoshop, Adobe, USA). Masson' trichrome stained sections, magnified at 30x, were used to easily distinguish the histological structures through their typical colours such as collagen fibres stained in dark and light blue(Miot & Brianezi,2010). First, in each

image, the epithelium and the fibrin clot were manually selected and excluded. The remaining pixels in the ROI_1 indicating the area of the granulation tissue, were measured and expressed as a percentage of the total pixels of the ROI_1. In order to quantify the collagen of the granulation tissue area, collagen pixel red green blue (RGB) values were recorded at 5 randomly selected points to obtain an average pixel RGB value representative of the collagen content. This RGB value was set as a threshold to select all the blue pixels per image using the Select > Color Range function with a "fuzziness" value of 70(Grasman *et al.*,2015). The thresholded pixels were expressed as a percentage of the granulation tissue area.

-***Inflammatory cells, Fibroblasts and Vessels:*** A 100-point grid region of interest(ROI_2) was overlaid on images of the central superficial area in H&E stained slides magnified at 200x(Image J). Points were counted coinciding with the following components: inflammatory cells, fibroblasts and blood vessels.

2.4.4- Immunohistochemical analysis

In order to detect the proliferative pattern of oral keratinocytes in the epithelial compartment, antibody against rabbit anti Ki-67(ab15580, Abcam, USA) was used for heat-induced antigen retrieval with a developing at 4◦C overnight with secondary antibody kits and DAB as the chromogen according to the respective protocol. The negative control was achieved staining the slide without treatment of the primary antibody to evaluate false positive staining.

The number of positive keratinocytes was calculated using a region of interest(ROI_3)(Safferling *et al.*,2013) (Fig.1).

2.4.5. Statistical analysis

Mean and standard deviation values of clinical and histomorphometrical parameters were calculated for each experimental group. Kruskal-Wallis test with Dunnett's test for post-hoc analysis were used to quantify differences between groups with $p \leq 0.05$ considered as significant. Sample size calculation was based on intra-individual evaluation of wound closure with a difference of 60%, SD 2-8%(Ayvazyan *et al.,*2011), a significance level of 5% and 80% power, leading to a total animal number of at least 10.

3- RESULTS

3.1- Clinical wound area

Photographic recordings at PSD4, 8, 12 and 16 demonstrated a constant decrease of the wound areas over time in all groups(Fig.2a,b). From PSD8 to PSD16, group EGF1 displayed the smallest mean wound area, while the largest was observed in group EGF50.

At PSD4, mean wound areas were 70.51±4.43mm^2, 60.37±8.1mm^2, 62.51±3.36mm^2, 61.88±8.25mm^2 and 65.31±11.91mm^2 in groups SH, V, EGF1, EGF10 and EGF50 respectively.

At PSD8, the mean values of the defect areas were 52.01±13.16mm^2, 34.64±5.54mm^2, 29.89±14.93mm2, 31.71±9.38mm^2 and 58.73±25.51mm^2 in SH, V, EGF1, EGF10 and EGF50 groups respectively.

At PSD12, the groups SH, V, EGF1, EGF10 and EGF50 exhibited mean values of respectively 25.58±10.34mm^2, 22.01±13.9mm^2, 4.83±1.99mm^2, 15.83±10.84mm^2, 51.15±31.49mm^2.

At PSD16, the mean areas were 13.19±2.73mm^2, 17.56±12.89mm^2, 1.76±2.19mm^2, 7.04±5.08mm^2, 29.53±19.96mm^2 in SH, V, EGF1, EGF10 and EGF50 corresponding respectively to a closure of 83.2%, 77.6%, 97.8%, 91% and 62.4% of the initial wound area.

Differences between groups reached statistical significance at PSD8 for V, EGF1 and EGF10 when compared to SH and EGF50($p<0.05$). At PSD12 and PSD16, statistical difference was obtained between EGF50 and the remaining groups($p<0.05$).

3.2- Volumetric assessment of wound repair

The results of the volumetric measurements, summarized in Table 1 and illustrated in Fig.3, demonstrated that a similar tissue volume was harvested from the palatal mucosa in all groups at baseline(i0-i1)(p>0.05). At PSD4(i1-i2), tissue formation was observed in all defects with a mean volume of 98.09±23.51mm^3, 119.63±28.3mm^3, 135.84±mm^3, 126.26±mm^3 and 103.89±mm^3 in respectively SH, V, EGF1, EGF10 and EGF50 groups. At PSD8(i1-i3), however, a decrease of tissue volume was found in all groups but EGF10. The mean volumes at day 8 were 76.02±20.45mm^3, 94.49±19.33mm^3, 116.38±26.76mm^3, 133.12±23.79mm^3, 81.78±34.59mm^3 in respectively SH, V, EGF1, EGF10 and EGF50 groups. Subsequently, the highest tissue volumes were found at PSD12(i1-i4) in group EGF10(143.8±19.4mm^3), and at PSD16(i1-i5) in group EGF1(136.6±23.12mm^3), closely followed by EGF10(136.28±37.18mm^3). For both time points, the lowest was displayed by EGF50 group(74.74±51.23mm^3 at PSD12, 71.71±12.24mm^3 at PSD16). Statistical significance was observed at PSD8(i1-i3) between EGF10 and all groups(p<0.05) except EGF1; and at PSD12(i1-i4) and PSD16(i1-i5) for EGF50 as compared to EGF1 and EGF10(p<0.05).

3.3- Histological analysis of healing kinetic

EPITHELIUM LAYER

DAY 8 POST-SURGERY: Compared with SH, V and EGF50 where sparse and bare epithelial layers were formed at the wound site, epithelial cells showed a clear tendency to migrate from the edges of normal mucosa toward the centre of the wounds in groups EGF1 and EGF10(Supplement Fig. 1). As illustrated in Fig.2c, the histomorphometrical measurements showed that the wound gap was the smallest in groups EGF1 and EGF10. Mean values were 6.37±1.38mm, 4.75±0.76mm, 4.22±1.57mm, 4.67±1.35mm and 6.9±0.81mm in respectively groups SH, V, EGF1, EFG10 and EGF50. In addition, EGF1 displayed the longest mean length of new epithelium. The measurements provided mean values of 3.08±1.2mm,

3.85±1.25mm, 5.53±1.3mm, 4.82±0.19mm and 3.1±1.31mm in respectively SH, V, EGF1, EFG10 and EGF50. Statistical difference was observed for the mean wound gap in V, EGF1 and EGF10, in comparison with EGF50 (p<0.05). Similarly, the difference between epithelium length in EGF1 and EGF10 compared to the other groups was significant(p<0.05).

DAY 16 POST-SURGERY: Wounds were observed to reach full closure by reepithelialization in the majority of the samples in EGF1 and EGF10(5/6 and 10/12 respectively) while the epithelium was still not completely formed at the centre of the wounds in SH, V and EGF50(Fig.4a). This was verified by the wound gap measurement in groups EGF1 and EGF10 showing the fastest decrease upon PSD16(6.5 and 5.3 folds respectively). The mean values of new epithelium length were 7.21±1.63mm, 7.14±1.43mm, 12.94±1.44mm, 11.48±2.49mm and 6.58±2.39mm in SH, V, EGF1, EGF10 and EGF50 respectively. For both histomorphometrical parameters, EGF1 and EGF10 displayed significant differences when compared to group SH and EGF50(p<0.05).

SUB-EPITHELIAL LAYER:

DAY 8 POST-SURGERY: In all groups, the subepithelial region showed fibroblasts migrating from the wound bed and granulation initiating, as the defect site was replaced with regenerating tissue. In the magnified images of the middle region, connective tissue appeared heavily infiltrated with inflammatory cells between which small vessels could be detected with a sporadic distribution of few fibroblasts(Fig.4a,Supplement Fig.2). The histomorphometrical analysis showed no differences of vessels proportion between groups. By contrast, EGF10 group harboured the smallest inflammatory infiltrate among all groups(38.9±11%) as well as the highest proportion of fibroblasts(22.9±13%, p<0.05)(Fig.4b). The amount of collagen and granulation tissue was the highest in the subepithelial layer of defects belonging to EGF10 group(37.9±16.3% and 42.1±8.8%

respectively)(Fig.5). The differences between EGF10 and the other groups for the proportions of collagen, granulation tissue and inflammation reached statistical significance(p<0.05).

DAY 16 POST-SURGERY: Examining the central subepithelial region of wounds belonging to EGF1 and EGF10, collagen fibres appeared thicker with a corrugated structure, whereas highly disordered in SH, V and EGF50, as evidenced with the H&E staining(Fig.4a). The histomorphometry showed a significantly higher deposition of collagen, faster regression of granulation tissue and lower proportion of inflammatory infiltrate in EGF1 and EGF10 in comparison with SH, V, and EGF50(p<0.05)(Fig.4b and Fig.5). The collagen area amounted 33±8.8%, 37.2±3.1%, 57.7±5.4%, 57.5±15.2% and 44.4±15.9% in respectively SH, V, EGF1, EGF10 and EGF50. The area of granulation tissue represented 45.5±10%, 45.2±4.2%, 21.3±5.2%, 24.9±14.5% and 27.9±11.5% in respectively SH, V, EGF1, EGF10 and EGF50. The inflammatory infiltrate proportion reached 70.6±11.8%, 42±25.4%, 26.5±23.7%, 13.6±14.6% and 47±31.2% in respectively SH, V, EGF1, EGF10 and EGF50. In addition, fibroblasts and vessels proportions remained the highest in EGF10 group with statistical difference as compared to the other groups(p<0.05).

3.4- Keratinocytes proliferative activity

As shown in Fig.6.a, few positive nuclei along the basal cell layer could be observed in the edges of the wounds from SH, V or EGF50 at PSD8 and PSD16. However, a clear increased expression of Ki67 within basal and immediate supra-basal cells was seen from PSD8 to PSD16 in EGF1 and EGF10. This was confirmed by the histomorphometrical analysis demonstrating a significantly higher keratinocytes proliferation in these groups at PSD16(p<0.05). The immunoreactivity for Ki67 was 399.2±165.1cells/field, 579.4±180.9cells/field, 921.9±202.3cells/field, 1362.9±564.9cells/field and 340.1±315.51cells/field in SH, V, EGF1, EGF10 and EGF50 respectively.

4- DISCUSSION

There have been through and accelerated efforts to improve oral wound healing. These endeavours have been motivated by the need for a hastened soft tissue healing following periodontal and peri-implant surgery. Although the oral mucosa is endowed with the capacity to repair itself, closure is anticipated to be significantly delayed when surgical wounds are readily exposed to oral bacteria and left to heal by secondary intention. Because complications of open wounds may hamper the treatment outcomes, it is believed that surgical wounds healing by secondary intention require local care(Greenstein *et al.*,2008). This study provides a hitherto novel approach for boosting the innate healing potential by which rhEGF is engaged in a productive repair of oral mucosa wounds.

The hypothesis of the present study encompasses not only the study of the rhEGF biological potential, but also the screening of the clinically relevant doses. A total of fourteen dogs was employed to discriminate the dose-dependent effect of rhEGF on oral wound repair. Considering the paucity of the data for the oral mucosa(Kim *et al.,*2017), three concentrations were selected in accordance with the literature addressing the treatment of skin wounds in humans(Brown *et al.,*1989; Wang *et al.,*2002; Fernández-Montequín *et al.,*2009; Martí-Carvajal *et al,*2010) or animals(Hong *et al.,*2006; Gainza *et al.,*2015) with rhEGF. Then, rhEGF incorporated in an ointment vehicle was tested in a discriminating pre-clinical model. The experimental standardized wound size, fixed wounding protocol, and the use of canine palatal mucosa as a wounding substrate allowed to overcome some of the challenges associated with in vivo wound healing studies, that is, the lack of apposite murine wound healing model for oral soft tissues. The morphological features of oral epithelium and molecular patterns in connective tissue of the healing oral palate from dog are the most similar to humans as compared to other species including rodents and pigs(Sa *et al.*,2016;2017). Here, the harvested tissues with a mean thickness varying between 1.32mm and 1.49mm, resulted in defects that included epithelium, lamina propria and submucosa. Sampling was performed at one and two weeks after excisional

wounding, time points at which the healing masticatory mucosa exhibited typical signs of erythema/oedema or wound closure respectively(Häkkinen *et al.,*2012). Given the excisional nature of the wounds, healing requires the closure of the wound site by formation of new connective tissue and coverage by epithelium, events that might be impaired by direct insults from the oral commensal flora(Nooh *et al.,*2003). Growth factors, such as EGF, are poised to improve wound repair in the present setting of acute excisional injuries(Davidson *et al.*,1993).

Consistent with this assumption was the healing in wounds topically treated with rhEGF 1μg/g and 10μg/g, that is characteristic of a regenerative response including efficient resolution of inflammation, promotion of an extra-cellular matrix(ECM)– rich granulation tissue, and a superior rate of re-epithelialization(Gurtner et al.,2008). A prime example is the rapid closure of the excisions receiving topical preparations in EGF1 and EGF10 as evidenced clinically(gross wound area) and confirmed histologically(wound gap), and may be attributable to a plethora of biochemical events downstream of the binding of EGF to its transmembranic receptors(Nickoloff *el al.,*1988; McCawley *et al.*,1998; Hashimoto *et al.,*2000; D'Alessio *et al.*,2008). In the same vein, the analysis of the proliferative activity as reflected by Ki-67, a protein produced in mitosis G1 and S phases, was found to be superior in sites receiving 1μg/g and 10μg/g of rhEGF as compared to EGF50 and controls at PSD8 and PSD16. In contrast with the impaired wound closure observed in EGF50, the overall quicker reconstruction of the epithelium in EGF1 and EGF10 confirms previous reports using settings of skin wounds in pigs(Hong *et al.*,2006) or humans(Yang *et al.,*2016).

Once the epithelial barrier has been re-established, remodelling of the granulation tissue concomitant with the deposition of cross-linked collagen fibres are key events in the course of oral mucosa healing(Weinreb *et al.,*2015). Comparably to previous studies, the significant production of hypercellular granulation tissue(Kondo *et al.,*2012) at 8 days as well as the substantial deposition of highly-organized collagen(Gainza *et al.,*2015) at 16 days illustrate

herein the faster healing in rhEGF-treated sites, particularly with the 1µg/g and 10µg/g regimen at the opposite of the 50µg/g concentration. Through the activation of mitogen-activated protein kinase-dependent pathways(Smith *et al.,*2009), not only EGF stimulates gingival fibroblasts to remove fibrin clot (Chiquet *et al.,*2015), but also activates matrix metalloproteins, major ECM-proteolytic enzymes involved in the healing of human acute gingival wounds (Smith *et al.,*2009). Surprisingly, while fibroblasts are poised to reduce in number during ECM remodelling, these cells were more numerous at PSD16 in sites treated with the concentrations 1µg/g and 10µg/g, seemingly due to the EGF proliferative effect on these cells as previously reported(Irwin *et al.,*1994). Altogether, these histological events were translated clinically by the highest volumes of regenerated tissues within wounds receiving 1µg/g and 10µg/g up to PSD16, despite the volume diminution ascribed to the swelling subside as observed for the other groups.

The rapid onset and regression of the inflammatory response has a crucial role in coordinating the process of wound healing(Gurtner *et al.,*2008). Less inflammatory cells were histologically evidenced in EFG1 and EGF10 at the opposite of SH and EGF50 where persistent inflammatory response up to PSD16 was found. The finding that exogenous EGF attenuates inflammation is in line with an existing evidence accumulated from preclinical(Heck *et al.,*1992; Kim *et al.,*2010) and clinical trials(Shin *et al.,*2015), and may be linked to a substantial decrease of histamine release by monocytes(Babül *et al*.,2004). Inflammatory cells secrete essential cytokine and growth factors necessary for angiogenesis and stromal cell migration(Eming *et al.,*2007). With regard to a recent report demonstrating a substantial increase in vascular endothelial growth factor (VEGF) synthesis by human gingival fibroblasts exposed to EGF in vitro(Pansani *et al.,*2017), the elevated neovascularization EFG1 and EGF10 is not a surprising finding and strongly suggests the ability of rhEGF to promote oral mucosa wound healing by facilitating angiogenesis in addition to an earlier resolution of inflammation(Gainza *et al.,*2015).

However, an unexpected outcome was the failure of wounds in EGF50 to reepithelialise, which combined with their persistent inflammatory response suggests the development of chronic lesions(Koivisto *et al.,*2012). Soft tissue swelling and excessive inflammatory response were reported in wounds treated with highly-dosed regimen of growth factors(Zara *et al.,*2011). In animal settings of skin wounds, topical treatment with 10µg/ml EGF delayed the closure of acute lesions(Breuing *et al.,*1997) while less proliferative activity of keratinocytes was depicted for doses of rhEGF superior to 10µg/g(Hong *et al.,*2006). It is believed that a downregulation of epidermal growth factor receptors occurs when cells are maximally stimulated with exogenous EGF, ultimately leading to the deceleration of the wound healing process(Mathers *et al.*,1989). The decrease of lipocortin activity resulting in prostaglandin upregulation and inflammation exacerbation was also reported to explain the detrimental effect of EGF's high dosages(Tripathi *et al.*,1990). The caveat is that dose-associated events might also include neoplastic transformation(Zeng *et al.,*2014), the short duration of this study, however, limits any conclusion on the effects of high doses of rhEGF as well as the maturation of healing tissue for a longer span of time.

5- CONCLUSION

Within the limitations of the present study, the observations herein demonstrate the efficacy of topically administrated human recombinant EGF in promoting early healing of open soft tissue wound in dogs. The results suggest that concentrations of 1 and 10ug/g in ointment accelerates wounds closure due to the increase of keratinocytes proliferative activity in the epithelial layer; and fastens neovascularization and inflammation regression in the subepithelial compartment. In contrast, a persistent inflammatory response and a delay of the wound closure were observed in wounds treated with the concentration 50ug/g of rhEGF.

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TABLES

***Table 1*:** Showing the outcomes of the volumetric analyses for the control (negative control; SH, vehicle; V) and the test groups (EGF1, EGF10 and EGF50). Mean, median and standard deviation (SD) is given for each parameter.

	Outcomes of volume measurements (integrated distances, mm^3) comparing the situation before surgery (i0), 4 days after surgery (i2), 8 days after surgery (i3), 12 days after surgery (i4) and 16 days after the surgery (i5) with the situation directly after surgery (i1)														
	i0-i1			i1-i2			i1-i3			i1-i4			i1-i5		
	Mean	Median	*SD*	**Mean**	Median	*SD*	**Mean**	Median	*SD*	**Mean**	Median	*SD*	**Mean**	Median	*SD*
SH	**-151.3**	-140.93	*47.58*	**98.09**	90.9	*23.51*	**76.02**	65.65	*20.45*	**87.39**	66.87	*42.8*	**85.23**	68.3	*38.97*
V	**-157.32**	-160.22	*45.78*	**119.63**	125.8	*28.3*	**94.49**	91.39	*19.33*	**89.82**	90.84	*14.47*	**77.73**	102.27	*12.24*
EGF1	**-163.25**	-164.84	*34.17*	**135.84**	140.09	*41.08*	**116.38**	123.49	*26.76*	**124.32** ^{b}	124.8	*30.84*	**136.6** ^{b}	129.03	*23.12*
EGF10	**-147.2**	-152.2	*38.79*	**126.26**	130.2	*57.9*	**133.12** ^{a}	130.62	*23.79*	**143.8** ^{c,d}	137.6	*19.4*	**136.28** ^{b}	131.4	*37.18*
EGF50	**-152.01**	-146.82	*36.78*	**103.89**	105.73	*30.52*	**81.78**	87.6	*34.59*	**74.74**	86.3	*51.23*	**71.71**	66.02	*53.76*

a: significant difference with SH, V and EGF50 groups (p<0.05).
b: significant difference with EGF50 (p<0.05).
c: significant difference with SH (p<0.01).
d: significant difference with EGF50 (p<0.001).

FIGURE LEGENDS

Figure 1: a-d: Surgical procedure, rhEGF administration and follow-up**.** Using a prefabricated stent ***(a)***, punch biopsies were harvested creating 6 defects on the hard palate mucosa of each dog ***(b)***. An equivalent volume (≈240 mm^3) of ointment (vehicle, 1, 10 and 50µg/g) was transferred to the stent using a spatula on the location of each defects. The stent was then applied on the upper jaw and an intimate contact of both jaws was maintained to immobilize the stents for 10 minutes. In the *Spontaneous Healing* group, the same procedure was performed while no ointment was applied on the stent ***(c)***. Healing was monitored using clinical photographs and impressions ***(d)*** (Bar=5mm)***.***

e-g: Clinical, three-dimensional and histological analyses. On photographical recordings of the wounds before, immediately after surgery, 4, 8, 12 and 16 days following surgery, the wound area (mm^2) was calculated as the surface area covered by unkeratinized tissue (dashed area) ***(a)***. Tissue volume variations were evaluated in regions of interest (ROI_V) on superimposed scans of digitized casts at the different time-points (Blue: immediately after surgery, Grey: at post-surgery day-4) ***(b)***. Histological analysis was performed using ROI_1 positioned at the centre of the biopsy along its surface, a rectangular-shaped ROI_2 (8mm/1.5mm) and ROI_3 encompassing the epithelium layer (12mm length segment divided in five equivalent fields of x200 magnification).

Figure 2: a- Representative clinical photographs of wounds healing following surgery, at post-surgery days 4, 8, 12 and 16. ***b-*** Remaining wound area from surgery to post-surgery day 16. ***c-*** Histological evaluation of epithelium length (left) and wound gap (right).

(● : Spontaneous healing (SH); ■ : Vehicle (V); ▲ : EGF1; ▼ : EFG10; ◆ : EGF50 ;
a: significant difference with V, EGF1 and EGF10 ($p<0.05$);
b: significant difference with V, EGF1 and EGF10($p<0.01$);
c: significant difference with SH, V, EGF1 and EGF10 ($p<0.01$);
d: significant difference with SH, V, EGF1 and EGF10 ($p<0.05$);
†, ‡: significant difference with V, EGF1 and ($p<0.05$);
Significance level set at *: $p<0.05$, ***: $p<0.01$,****: $p<0.001$;
Bar=5mm)

Figure 3: Soft tissue volume alterations in defects receiving vehicle (V), 1µg/g (EGF1), 10µg/g (EGF10) and 50µg/g (EGF50) of rhEGF or spontaneously healing (SH) between immediate post-surgery and day 16. Occlusal view of the regions of interest (top row) and their transversal sections (lower row) are illustrated. The thickness differences in the soft tissues (mm) are displayed on the coloured scale at the bottom of the figure. Blue areas represent a decrease in thickness, green areas a comparable thickness and yellow and red areas an increase in thickness. (Dashed line: reference for transversal section of the region of interest; Black bar=2.5mm)

Figure 4: a- Representative H&E staining of the palatal mucosa unwounded or 8 and 16 days following surgery, illustrating the healing process at the centre of the defects (see Supplement Fig. 2). Boxed areas are high magnifications (x400) shown for unwounded (on the right) and wounded tissues at days 8 and 16 (below). Asterix indicates vessel-like structures, dotted lines represent boundaries between fibrin clot and granulation tissue. ***b-*** Histomorphometric

quantification of the proportion of fibroblasts, vessels and inflammatory cells-like structures in the central superficial area of the wounds 8 and 16 days following surgery (region of interest ROI_1) (Black bar= 100μm; yellow bar= 20μm).

(SH: spontaneous healing; V: vehicle)
a: significant difference with SH, V, EGF1 and EGF50 (p<0.05);
b: significant difference with SH, V, EGF1 and EGF50 (p<0.01);
c: significant difference with SH, V and EGF50 (p<0.05);
d: significant difference with SH, V and EGF1 (p<0.05)
***:** significant difference between day 8 and day 16 (p<0.05);
******: significant difference between day 8 and day 16 (p<0.01).

Figure 5: Graphical representations of granulation tissue and collagen proportions at post-surgery days 8 and 16 in wounds healing spontaneously (SH) or receiving vehicle (V), 1μg/g (EGF1), 10μg/g (EGF10) and 50μg/g (EGF50) of rhEGF ointments.

(**a**: significant difference with SH, V, EGF1 and EGF50 (p<0.05);
b: significant difference with SH, V and EGF50 (p<0.05);
***:** significant difference between day 8 and day 16 (p<0.05);
******: significant difference between day 8 and day 16 (p<0.01).

Figure 6: a- Immunohistochemical staining of normal (top row) and healing palatal mucosa (spontaneously healing, NC; vehicle, V; rhEGF 1μg/g, rhEGF 10μg/g and rhEGF 50μg/g groups) with antibody against Ki67. Figures illustrates proliferative activity of basal keratinocytes at the epithelium of wound edges (Bar=50μm). ***b-*** Graphical representation of Ki67 positive cell number per field. Dashed red line indicates the mean number of Ki67 positive cells per filed in the epithelium of unwounded mucosa.

Supplement Figure 1: Wound closure and reepithelialization at post-surgery day 8 illustrated by Masson's trichrome staining of oral mucosa wounds from spontaneously healing (NC,***a***), vehicle (V, ***b***), EGF1 (***c***), EGF10(**d**) and EGF50 (***e***). Dashed line represents epithelium length, black arrow indicates non-wounded tissue margin, white arrow shows the extremity of wound epithelium and ulcer margin. (Bar= 500 μm).

Supplement Figure 2: Representative H&E staining of oral mucosa sections at 8 days (***A***)and 16 days (***B***) following surgery. Clinical wound repair is represented, and histological healing process is shown at the left (***a, d, g, j, m***), centre (***b, e, h, k, n***) and right of the wounds. White dashed lines on histological sections indicate the boundaries between the wound and the surrounding normal tissue. Magnified view (x100, ***p-t***) of the regions in black pane are presented on the left column of ***A*** and ***B***. Asterix indicates vessel-like structures, black dotted lines represent boundaries between fibrin clot and granulation tissue (Black bar= 100 μm, white bar= 200μm).